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ENHANCEMENT OF IN VITRO GRANULOCYTE-MACROPHAGE COLONY FORMATION BY NORMAL HUMAN SERUM OR PLASMA

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Research was conducted according to the principles enunciated in the "Guide for Laboratory Animal Facilities and Care," prepared by the National Academy of Sciences - National Research Council.

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Irreversible bone marrow damage is the principal etiologic factor causing death after exposure to ionizing radiation. Therefore, methods of improving recovery of bone marrow function are highly critical. The present study is part of a research program exploring methods of improving that recovery. Enhancement of human and murine bone marrow colony formation in agar gel was studied with respect to species specificity, ability to enhance various colony stimulating factors, dose-response

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relationship, the abilities of nonhuman sera to enhance colony formation, and mechanism of action. Normal human serum enhanced colony formation by both human and murine marrow cells. Murine colony formation was enhanced by normal human serum when several different types of colony stimulation factors were used. Enhancement by nonhuman sera was absent or much less than by normal human sera. Optional concentrations of normal human serum for enhancement were 7.5 percent and 13.5 percent (volume/volume) in the murine and human systems respectively. Normal human serum increased the slope of the curve describing the number of colonies versus colony stimulating factor concentration suggesting that it modifies the action of the latter. Preincubation of marrow with serum did not result in enhancement. Nonhuman colony stimulating factor did not decrease enhancement by normal human serum of human marrow colony formation. This work suggests that current methods of treatment of radiation-induced bone marrow damage can be significantly improved.

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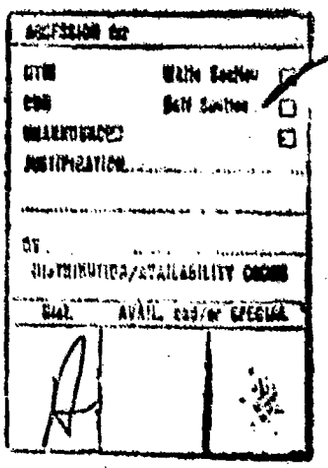

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INTRODUCTION

Mouse or human bone marrow cells form visible colonies when cultured in agar gel in the presence of a source of colony stimulating activity (CSA).[2] The curve which describes the relationship between CSA concentration and colony number is sigmoid and the amount of CSA used to stimulate colony formation is ordinarily on the plateau of the dose-response curve.[9] It is of interest, therefore, that certain materials are capable of increasing the number and size of colonies formed in vitro in the presence of an optimal CSA stimulus. The enhancing activity of red blood cell hemolysates has been described and characterized in part.[4,10] More recently, the enhancing effect of normal human serum (NHS) on murine colony formation in vitro has been reported.[8] In the present report, we describe the ability of NHS or plasma to enhance in vitro colony formation by either murine or human bone marrow cells. We have studied this enhancing activity with respect to species specificity, ability to enhance various colony stimulating factors, dose-response relationship, and the abilities of non-human sera to enhance colony formation. In addition, we have attempted to determine whether NHS acts independently or in conjunction with the colony stimulating factor (CSF) to produce its effect and to provide some preliminary physical characterization of the active principles.

METHODS AND MATERIALS

Culture techniques. Murine bone marrow cells, usually 5×10^4 cells/culture plate, were dispensed in volumes of 1 ml of culture medium containing 0.3 percent agar over a 2-ml basal layer containing culture medium, 0.5 percent agar, and a source of CSA.

Enhancement of colony formation stimulated by different sources of CSA was studied. The source of CSA was either 10^6 normal human peripheral blood leukocytes per culture plate, an extract prepared from the uteri and placental membranes of pregnant mice (PMUE),[3] or mouse L-cell conditioned medium.

The culture media used was that described by MacVittie and McCarthy[6] containing CMRL 1066, 10 percent fetal calf serum, 10 percent trypticase soy broth, 5 percent horse serum, and 30 micrograms/ml of l-asparagine. This was prepared in a concentration of twice normal osmolarity and, together with cells and serum, diluted with an equal volume of either 1.0 percent (underlayers) or 0.6 percent (overlayers) agar.

Animals used to provide marrow were male and female Swiss Webster or B6D2F1 BR mice (Cumberland View Farms), 8-12 weeks of age.

Collection of bone marrow. Three to four milliliters of human bone marrow were collected in preservative-free heparin (Flow Laboratories, Rockville, Maryland) from consenting patients* in the course of diagnostic posterior iliac crest marrow aspiration. The red cells were allowed to settle by gravity and the nucleated cell-rich plasma collected. The cells were centrifuged at approximately 100 x g for 5-6 minutes and resuspended in 2-4 ml of culture medium (RPMI 1640). Murine bone marrow cells were flushed with culture medium from the femurs and tibias and utilized directly.

Preparation of human leukocyte feeder plates. Human peripheral blood leukocytes were obtained from consenting adults to provide a source of CSA in the agar culture system. The red cells were sedimented by gravity using 25 percent (v/v) of 3 percent dextran in Earle's salts (Flow Laboratories). The leukocyte-rich plasma was removed and the cells centrifuged (100 x g for 5-6 minutes) and resuspended in Hanks' balanced salt solution. The volume of cells required to provide 10^6 cells/2 ml in the final suspension was added to 1.0 percent agar (bacto, Difco) and double strength culture medium. Two milliliters of the latter were dispensed into 60-mm plastic petri dishes (Falcon Company, Oxnard, California) and allowed to gel.

Preparation of sera or plasma. Human, canine, and mouse blood was collected with or without preservative-free heparin. Human donors were asked to

* Informed patient consent was obtained in accordance with the guidelines specified by the Committee for the Protection of Human Subjects, National Naval Medical Center, Bethesda, Maryland

sign a standard consent form. Serum was collected and filtered through 0.45-μm filters (Nalge Company) and stored at -20°C. Human sera of various ABO and Rh types were used in different experiments. Horse serum and fetal calf serum were obtained commercially (Flow Laboratories).

Serum or plasma was added usually to the overlayers, but at times also to the underlayers, of the agar culture system. The final concentration described is based on the amount of serum in the total culture volume.

Mechanism of enhancement. Murine bone marrow colony number was determined in the presence of increasing concentrations of CSA (PMUE). Two curves were plotted showing this relationship with and without serum added to the culture plates. Demonstration that NHS modifies the concentration dependence of colony number of PMUE would suggest that NHS acts by enhancing CSF rather than merely to provide some supplementary growth stimulus.

The ability of NHS to enhance colony formation by merely preincubating the cells with serum was studied. Murine bone marrow cells were incubated for 1 hour in suspension with either 25 percent (v/v) NHS, fetal calf serum, or culture medium alone (RPMI 1640). Each suspension was centrifuged and the cells resuspended in fresh RPMI 1640. The number of cells per ml in each fraction was then redetermined using a Coulter model "F" cell counter and the appropriate dilutions made to reequate the cell counts. Each fraction was then cultured. In addition, NHS (7.5 percent v/v) was added to a portion of the cells preincubated with RPMI alone.

In order to examine the possibility that the enhancing factor in NHS might combine with or be absorbed to CSF extracellularly, human bone marrow cells (2×10^5 per culture plate) were cultured in the presence of NHS (7.5 percent v/v) with and without added PMUE. PMUE is capable of stimulating colony formation by mouse but not by human bone marrow cells. It is known, however, that colony formation by mouse bone marrow is enhanced by NHS. Hence, if PMUE and NHS interact or combine extracellularly, this might reduce the quantity of NHS available to interact with human CSF provided by the human

peripheral blood feeder leukocytes. Thus, a reduction in the enhancement of human colony formation in the presence of PMUE would suggest extracellular interaction of the enhancing factor(s) and CSF.

Physical characterization. NHS was tested for enhancing activity before and after dialysis for 3 days against distilled water or phosphate-buffered saline. The effect of freezing and of heating to 56°C for 1 hour was also determined.

Evaluation of experimental results. Colonies estimated to contain 50 cells or more were counted. At least three replicate culture plates were generally employed for each experimental condition.

Data were analyzed statistically by making specific individual comparisons using Student's "t" test for unpaired data. Colony numbers were assumed to adhere to a Poisson distribution. Differences were considered to be statistically significant when "P" values exceeded 0.01.

RESULTS

Enhancement of human and murine colony formation by normal human serum (NHS). Normal human serum enhanced in vitro colony formation in the agar culture system by both human and murine bone marrow cells (Table 1). Both the number and size of colonies were increased. Serum alone did not

Table 1. Enhancement of Human and Murine In Vitro Bone Marrow Colony Formation by Normal Human Serum (NHS)

	Without serum	With serum		Δ%
No. Colonies ± S.E.M. Per 2×10^5 Human Bone Marrow Cells Stimulated by Human Peripheral blood Leukocytes	14.2	NHS MALE	28.0 ± 2.5	98
		NHS FEMALE	30.0 ± 1.2	111
		FCS	23.0 ± 1.5	62
No. Colonies ± S.E.M. Per 5×10^4 Murine Bone Marrow Cells Stimulated by Human Leukocytes	59.4	NHS MALE	130.7 ± 4.7	120
		NHS FEMALE	149.3 ± 8.4	151
		FCS	84.0 ± 1.7	41

stimulate colony formation. There were no differences in the enhancing abilities of human male and female sera or of serum and plasma.

Enhancement of colony formation in the presence of various colony stimulating factors. Human serum enhanced murine colony formation stimulated by normal human peripheral blood leukocytes, pregnant mouse uterus extract (PMUE), and mouse L-cell conditioned medium (Table 2). A 0.3 percent NHS control was used to exclude the possibility of nonspecific antigenic stimulation.

Table 2. Effect of Normal Human Serum (NHS) on Colony Formation by Mouse Bone Marrow Cells Using Different Colony Stimulating Factors

	Number of Colonies Per 5×10^4 Murine Bone Marrow Cells		
CSA Source	Control (0.3% NHS)	10% NHS	Δ%
PMUE	47.0	194	313
L CELL CM	72.0	179	148
HUMAN LEUKOCYTES	28.0	57	104

At the 0.3 percent NHS concentration, there was no detectable enhancement (Figure 1). Enhancement was most evident when control colony number was greatest. Thus, while enhancement of feeder leukocyte colony stimulating activity was approximately 100 percent, that with PMUE or L-cell conditioned medium was 150-300 percent (Table 2). This suggests that the action of human serum may be to enhance CSF rather than merely to provide a supplementary growth stimulus.

Dose-response relationship. Normal human serum enhanced colony formation by mouse bone marrow cells in a range of concentrations between 1 percent and 15 percent. The dose-response relationship for PMUE-stimulated

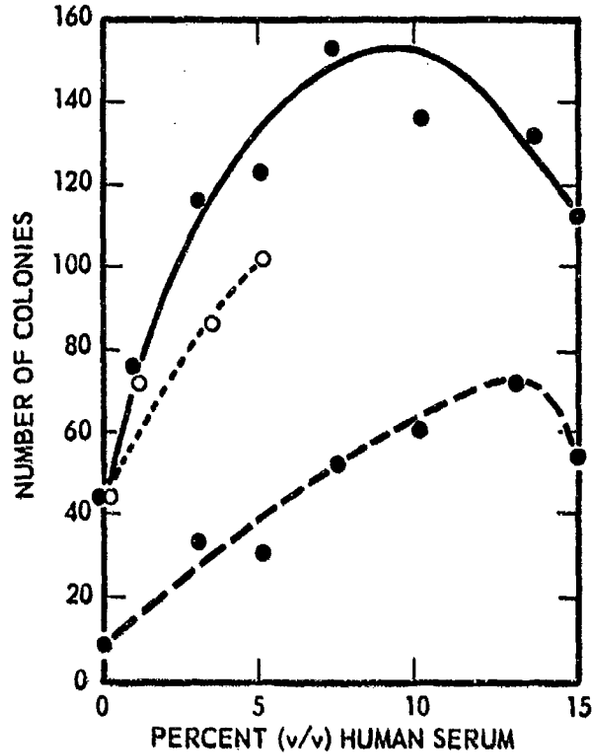

Figure 1. Dose-response curves for bone marrow colony formation in the presence of NHS. •—• Dose-response curve for murine bone marrow colony formation using PMUE as a source of CSA. •---• Dose-response curve for human bone marrow colony formation using human peripheral blood leukocytes as a source of CSA. o---o Partial dose-response curve for human bone marrow colony formation stimulated by a mononuclear cell population of human peripheral blood leukocytes.

murine bone marrow colony formation and NHS concentration is presented in Figure 1. Maximal enhancement was seen at a concentration of approximately 7.5 percent (v/v). The configuration of the dose-response curve for number of human bone marrow-derived colonies versus percent NHS was similar to that of murine marrow. A peak was seen at 13 percent serum with a decrease in colony number at the single higher concentration. Recently, we have employed a mononuclear cell preparation obtained by the use of a Hypaque-Ficoll gradient, together with added phytohemagglutinin (10 micrograms/10^6 cells per 2 ml) as a

source of CSA. These feeder leukocyte plates stimulated a greater number of colonies using, however, a different donor of human bone marrow. The response to several concentrations of NHS is shown also in Figure 1.

Enhancement of colony formation by nonhuman serum. Dog, horse and fetal calf sera (FCS) were compared to human sera for their ability to enhance colony formation (Table 3). Mouse bone marrow was cultured over human feeder leukocytes; the concentration of serum used was 3.3 percent (v/v). In this experiment, dog, fetal calf serum, and horse serum enhanced colony formation only slightly (20-30 percent) while NHS produced approximately a 70 percent increment in colony number. The difference between the number of colonies formed by the controls and that in the presence of NHS was statistically significant ($P < 0.001$), while colony enhancement in the presence of nonhuman sera was not statistically significant ($0.05 < P < 0.1$).

Table 3. Enhancing Effect of Various Sera on Colony Formation by Murine Bone Marrow Cells

CSA SOURCE:	HUMAN LEUKOCYTES		PMUE			
	Number of Colonies Per 5×10^4 Cells ± S.E.M.	Δ%			Number of Colonies Per 5×10^4 Cells ± S.E.M.	Δ%
Control	51.2 ± 5.2	-	Control	(N)	75.3 ± 13.1	-
				(H)	66.3 ± 10.4	-
Control	60.7 ± 2.2	-	FCS	(N)	99.7 ± 3.1	41
				(H)	81.3 ± 12.7	15
FCS	67.8 ± 2.9	22	DOG	(N)	104 ± 9.3	47
				(H)	97.7 ± 5.8	38
DOG	69.0 ± 5.0	23	HORSE	(N)	104.7 ± 16.6	48
				(H)	96.7 ± 1.5	37
HORSE	72.8 ± 4.3	31	MOUSE	(N)	77.3 ± 5.0	9
				(H)	37.0 ± 3.0	23
NORMAL HUMAN	94.3 ± 5.7	71	NORMAL HUMAN	(N)	138 ± 14.7	95
				(H)	110 ± 10.5	55

(N) Non heated serum.
(H) Serum previously heated to 56°C for one hour.

When PMUE was used as the source of CSA to stimulate murine colony formation, NHS also enhanced to a greater degree than dog, horse, or fetal calf serum (Table 3). Untreated murine serum increased the size of individual colonies examined at 10 days but their number was actually decreased compared to

controls. In the presence of serum which had been heated to 56°C for 45 minutes the number of colonies equalled controls and, again, were larger in size (Table 3).

Mechanism of action. The addition of NHS (7.5 percent v/v) increased PMUE-stimulated colony formation by mouse bone marrow cells by 90-100 percent (Figure 2). The slope of the dose-response curve was greater in the presence of serum which is consistent with the concept that serum modifies the activity of CSF.

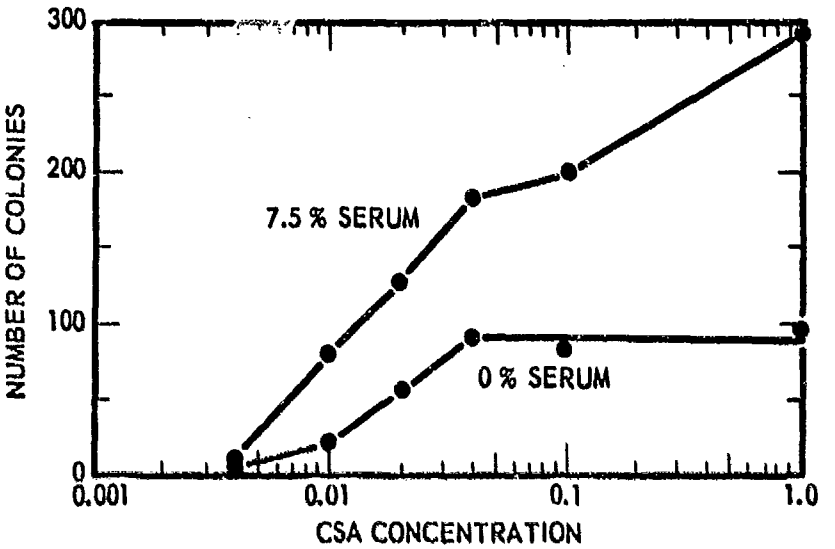

Figure 2. The effect of NHS on the CSA dose-response curve. Number of colonies per 5 x 10^4 murine bone marrow cells using PMUE as a source of CSA.

Preincubation of murine marrow cells with 7.5 percent (v/v) or 25 percent (v/v) NHS in suspension for 1 hour before culturing in agar did not result in enhancement of colony formation. In the same experiment, NHS in the underlayers enhanced colony formation by cells not preincubated with serum (Table 4). These results indicated that serum must be continuously present to enhance colony formation and that enhancement does not ensue as a result of one time exposure of the cell membrane to the serum factor(s).

Table 4. Effect of Preincubating Murine Bone Marrow Cells for 1 Hour with Serum before Plating in Agar

	Number of Colonies Per 5×10^4 Cells ± S.E.M.	
Control	Preincubated with Serum	Cultured with Serum
130±9.1	7.5% 138 ± 3.5	7.5 272 ± 10.4
	25 % 165 ± 9.1	25% 276 ± 20.2

Normal human bone marrow cells were cultured using human peripheral blood leukocytes as a CSA source and the effect of added PMUE on enhancement by NHS determined. Since it is known that human CSF (but not murine CSF) stimulates colony formation by both species, the molecular interaction of enhancing factor and an additional, but inactive, CSF source might reduce the degree of enhancement. In control cultures of human marrow stimulated by peripheral blood leukocytes, 44 ± 5.7 colonies (number ± S.E.M.) were obtained; with NHS (13 percent v/v) colony number increased to 130 ± 12.2. With both NHS and several dilutions of PMUE in the underlayers (undiluted, 1:1, and 1:9), 113 ± 16.5, 142 ± 9.8 and 137 ± 5.9 were obtained. Thus, the addition of PMUE in these concentrations did not influence enhancement by NHS.

Physical characteristics. The enhancing ability of NHS was preserved for over 6 months by freezing at -20°C. Its activity was not decreased by dialysis against phosphate buffered saline for 3 days but decreased after dialysis against distilled water for 3 days; (number of colonies per 5×10^4 PMUE-stimulated murine bone marrow cells) control 61.3 ± 2.7, with 2.5 percent NHS 126.3 ± 7.7, with 2.5 percent NHS after dialysis 81.4 ± 7.5. The decrease in activity following dialysis was -35.5 percent. Heating murine serum at 56°C for 1 hour increased the number of colonies from less than to equal to that of controls (Table 3). There was no apparent effect of heating on human serum.

DISCUSSION

We have studied the ability of normal human serum (or plasma) to enhance in vitro granulocyte-macrophage colony formation by both murine and human bone marrow. The addition of NHS to the culture system increased colony number and size by bone marrow cells from both species by 50-80.0 percent. Although murine serum failed to enhance the number of colonies formed, their size was increased in the presence of either untreated serum or serum which had been heated to 56°C for 30-45 minutes. Human sera enhanced colony formation stimulated by several sources of CSA and were more active in this respect than other nonhuman sera tested. The optimum concentration of NHS for enhancement of murine marrow colony formation was 7.5 percent (v/v); at higher serum concentrations, enhancement decreased, possibly because of other inhibitors present in serum.[5] The dose-response curve of human marrow colony number versus percent human serum was similar in appearance to that for murine marrow. NHS increased the slope of the curve describing colony number versus CSF_{PMUE} concentration suggesting that it may modify the activity of CSF. Serum was required to be continuously present to enhance colony formation. Enhancement of human bone marrow colony formation stimulated by human peripheral blood leukocytes was not inhibited by a murine source of CSA suggesting that enhancing factor(s) in serum did not combine extracellularly or interact irreversibly with CSF.

Factors which enhance hematopoiesis have been described in the past. Boggs et al.[1] described an effect of foreign plasma on improving postirradiation survival and endogenous spleen colony formation when such plasma was injected before irradiation. Red blood cell hemolysates have also been shown to enhance in vitro granulocyte-macrophage colony formation.[4,10] It has been shown also that foreign protein antigens may enhance colony formation.[7] An erythropoietin enhancing factor has been described in the serum of several patients with myeloproliferative disorders;[12] however, normal control AB serum did not display the enhancing effect. The ability of normal murine or human serum to enhance in vitro colony formation by murine bone marrow has recently been described.[8]

Van den Engh and Bol[11] have also reported on the ability of murine, postendotoxin serum to enhance colony formation by mouse marrow cells.

The mechanism of action of this enhancing activity is unknown. One might speculate that enhancing factor(s): (1) supplies some required metabolic factor necessary for cellular growth or division; (2) modifies target cell receptors for CSF, (3) recruits a secondary population of potential CFU-c responsive only to the combined presence of CSF and enhancing factors, or (4) modifies the CSF molecule to a more active form, either, for example, by supplying some cofactor, or by altering the molecular structure or configuration. The first possibility seems unlikely since not all sera enhance equally well. The second possibility also seems improbable since preincubation of the marrow cells with serum did not result in enhancement. The curve describing the relationship between colony number and PMUE concentration suggests that enhancing factor(s) may indeed modify the activity of the CSF molecule. If this is the case, it would not appear to do so by reacting extracellularly in an irreversible manner with CSF since PMUE did not inhibit enhancement of human, feeder leukocyte stimulated colony formation. Finally, enhancing factors may act to recruit a subpopulation of cells. Metcalf et al.[8] found no evidence for the latter using velocity sedimentation criteria. Williams and van den Engh,[13] however, have provided evidence to support the existence of such a subpopulation using the equilibrium density gradient technique.

In the present report we have described the ability of normal human serum to enhance colony formation by both human and murine bone marrow cells and have considered some of the possible mechanisms of action. Our observations with respect to the effect of NHS on murine colony formation are generally in agreement with those of Metcalf et al.,[8] and are, in addition, extended to human bone marrow culture. The observation that murine serum increased colony size without increasing colony number suggests the possibility that there may be different factors responsible for enhancement of the number and size of colonies.

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